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Modulation of Neutrophil Chemokine Receptors by *Staphylococcus aureus* Supernate

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In a previous study, we showed that *Staphylococcus aureus* supernate (SaS) is a potent agonist for both neutrophils and mononuclear cells. To further investigate the immunomodulating effects of SaS, the effect on different neutrophil receptors was studied. Expression of various neutrophil receptors, before and after treatment with SaS, was quantified by flow cytometry. We found that SaS treatment of neutrophils resulted in a specific and total downregulation of the C5a and the fMLP receptor, both serpentine receptors, while other receptors were totally unaffected. Since these two receptors are both involved in chemotaxis, we tested the effect of SaS in calcium flux and chemotaxis assays. We showed that preincubation with SaS abrogated the rise in intracellular calcium concentration upon triggering with fMLP and C5a. We also showed that SaS is a potent inhibitor of neutrophil chemotaxis towards fMLP and C5a, but does not interfere with chemotaxis towards interleukin-8. These findings indicate that *S. aureus* produces a virulence factor extracellularly, which impairs chemotaxis towards the infected site.

Staphylococcus aureus is one of the most common causes of bacterial infections in humans and animals. It produces serious wound infections, and in the hospital, it is one of the most important causes of nosocomial infections. *S. aureus* contains a variety of pathogenic factors that play a role in the disease processes and interfere with host defense. Cell wall components, such as protein A and peptidoglycan, are directly able to hamper humoral and cellular defenses. Protein A is capable of binding specifically to the Fc portion of the immunoglobulin G (IgG) molecule. Once bound to IgG, protein A may sterically block the interaction of IgG with its Fc receptor on phagocytic cells (16). Musher et al. described that both phagocytosis and chemotaxis were suppressed by prior preincubation of neutrophils with 2.5 μg of peptidoglycan of *S. aureus*/ml (14). The suppression of neutrophil function was independent of the presence of human serum but was abolished by rabbit antiserum to peptidoglycan. Cell wall components of staphylococci are also able to stimulate phagocytes. Peptidoglycan, teichoic acid, and lipoteichoic acid induce the release of cytokines by monocytes as previously described by several authors (1, 5, 9, 11, 12, 20).

S. aureus produces a variety of extracellular products, including cytolytic toxins that affect phagocytes as well as other cells. Most of the staphylococcal exoproducts are synthesized at the end of the logarithmic growth phase and are under the control of regulatory systems. The enterotoxins and toxic shock syndrome toxin 1 are superantigens which activate a subset of T cells by binding to major histocompatibility complex class II molecules on antigen-presenting cells and cross-linking to the T-cell receptor (10, 13). Proinflammatory cytokines are produced by T cells and monocytes following superantigen activation. Panton-Valentine leukocidin is leukotoxic for human and rabbit neutrophils and is able to induce the release of histamine, leukotriene B4, interleukin-8 (IL-8), and oxygen metabolites from human granulocytes. Panton-Valentine leukocidin-producing strains are mostly associated with infections as furuncles and abscesses as well as with severe pyodermic infections, such as dermonecrosis (9).

Delta toxin affects neutrophil leukotriene metabolism and, in conjunction with endotoxin, primes the oxidative burst activity (19). We recently described that additional products in staphylococcal culture supernates (SaS) stimulate phagocytes in whole blood generating tumor necrosis factor and IL-1β. We also showed that the cell-free supernate of *S. aureus* is able to upregulate CR3 expression on neutrophils, which was not caused by any of the known extracellular products (23).

The aim of this study was to further investigate the influence of staphylococcal cell-free supernates and their effects on neutrophil function. Since staphylococcal infections are often localized infections, like furunculosis, wound infection, arthritis, and endocarditis, we were particularly interested in the effect of extracellular products that interact with migration of neutrophil, a key event of the inflammatory response.

MATERIALS AND METHODS

Materials. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and recombinant C5a were purchased from Sigma Chemical Co., St. Louis, Mo. 4,4-Difluoro-4-bora-3a,4a-diaza-*S*-indacene (BODIPY)-labeled fMLP (BODIPY-*N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys), BCECF-AM [acetoxymethyl ester of bis-(2-carboxyethyl-5-(and-6)-carboxyfluorescein], and Fluo3-AM were from Molecular Probes (Eugene, Oreg.) Recombinant IL-8 was purchased from Pepro Techno Inc. (Rockey Hill, N.J.). Platelet-activating factor 16 (PAF-16) and ionomycin were purchased from Calbiogem-Novabiochem (San Diego, Calif.).

Monoclonal antibodies. The following monoclonal antibodies (MAbs) were used: 44a (anti-CD11b; CR3), 541 (anti-CD35; CR1), 60bca (anti-CD14), TS-1/22 (anti-CD11a), IB4 (anti-CD18), IV.3 (anti-CD32; FcRII), and W6/32 (anti-HLA class I) were obtained from the American Type Culture Collection as hybridoma cell lines, and the produced antibodies were purified over protein G-Sepharose (Pharmacia, Uppsala, Sweden); leu8-PE (anti-CD62L; L-selectin) and leuM5-PE (anti-CD11c) were from Becton Dickinson (San Jose, Calif.), and CLB-gran/1 (anti-CD16; FcRIII), CLB-gran/12 (anti-CD63), and CLB-gran/10 (anti-CD66) were from CLB (Amsterdam, The Netherlands). S 5/1 (anti-CD88; C5a receptor) was a gift from Otto Götze (University Göttingen, Göttingen, Germany), and SE-2 (anti-CD128w; 11-8R, Type A) and an anti-PAF receptor were from Alexis Corporation (San Diego, Calif.). Since no MAb for the fMLP receptor was available, the expression of this receptor was determined by binding of fluorescence-labeled fMLP. The secondary flourescein isothiocyanate (FITC)-labeled goat $F(ab')_2$ anti-mouse Ig was from Dako (Glostrup, Denmark).

SaS. *S. aureus* 1690 (a clinical isolate from a patient with a staphylococcal bacteremia) was cultured overnight at 37°C in Iscove's modified Dulbecco's

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medium (IMDM) synthetic medium (Life Technologies, Breda, The Netherlands), diluted 40-fold in fresh IMDM, and cultured for another 7 h under constant agitation. Bacteria were pelleted and the cell-free supernate was passed over a 0.2-μm-pore-size filter to remove residual bacteria. The supernate was dialyzed against phosphate-buffered saline (PBS) by using a dialysis membrane with a 10-kDa cutoff. After dialysis, SaS was aliquoted and stored at −20°C.

Cells. Blood obtained from healthy human volunteers was collected into tubes containing sodium heparin (Vacuette, Greiner, Alphen a/d Rijn, The Netherlands) as anticoagulants. Neutrophils were isolated as described by Troelstra et al. (22). For the isolation, heparinized blood was diluted 1:1 (vol/vol) with pyrogen-free PBS and layered onto a gradient of Ficoll (Pharmacia) and Histopaque (density, 1.119 g/ml; Sigma). After centrifugation for 20 min at 320 × *g*, the neutrophils were collected from the Histopaque phase. Cells were subjected to a brief hypotonic shock with water, washed, and suspended at 5×10^6 cells/ml in RPMI medium containing 0.05% human serum albumin (HSA) (CLB).

Receptor expression. A sample of 50 μl of either isolated neutrophils (5×10^6 cells/ml) or whole heparinized blood was mixed with different dilutions of SaS in a 1:1 (vol/vol) ratio and incubated for 30 min at 37°C under constant agitation. Samples were put on ice to terminate the interaction of SaS with the cells, and subsequently, the samples were incubated for 30 min on ice with 5 μl of MAbs at optimal concentration or 10 μM BODIPY-fMLP. Thereafter, whole-blood samples were treated for 5 min with flourescent-activated cell sorter (FACS) lysis buffer (Becton Dickinson), centrifuged, and washed once with RPMI-HSA, while isolated neutrophils were washed twice with buffer. Samples were either directly fixed with 0.5% paraformaldehyde in PBS or incubated with F(ab')2 FITC-labeled goat anti-mouse-Ig for another 30 min on ice. These samples were washed once more and also fixed with 0.5% paraformaldehyde. Controls of unlabeled cells and cells with only the secondary FITC conjugate were included. All samples were analyzed on a FACScan flow cytometer (Becton Dickinson), and the mean fluorescence was determined from 5,000 neutrophils after proper gating of the cells by forward and sideward scatter parameters. The expression of the different antigens was expressed relative to that of cells incubated in control buffer.

Measurement of calcium fluxes. Cells were loaded with 2 μM Fluo-3-AM in RPMI-HSA for 20 min at 37°C under agitation, washed twice with buffer, and suspended to 10^6 cells/ml in RPMI-HSA. Preliminary experiments were done to determine the optimal concentrations of the various chemoattractants for an adequate calcium flux. Chemokine-induced calcium fluxes are very rapid and transient, and therefore, from each sample, repetitive measurements of 2,000 events that required an average time of 10 s for sampling were done and saved to disk before the next acquisition. Each sample of 500 μl of cells was first measured for their fluorescence (at 530 nm) to determine the basal calcium level. Next, 5 μl of 100-fold-concentrated reagent was added while vortexing and quickly placed in the sample holder to start the timed series of measurements. For receptor desensitization, the labeled neutrophils were first incubated for 10 min at room temperature with SaS, fMLP, C5a, or buffer and subsequently stimulated with agonist. Samples were analyzed after gating the neutrophil population, thereby excluding cell debris and background noises. The calcium flux response of the cells for each time point was expressed as a relative value compared to the basal unstimulated level of that individual sample, and no conversions to actual calcium concentrations were done.

Chemotaxis. Chemotaxis of neutrophils towards chemoattractants like C5a, fMLP, and IL-8 was determined by using fluorescent labeled neutrophils that migrated through a membrane fitted into an insert Transwell system (Costar) containing a prewetted 3-μm-pore-size polycarbonate filter. Therefore 5×10^6 neutrophils were first labeled with 3.3 μM BCECF-AM for 20 min at room temperature, washed twice, and resuspended in RPMI-HSA. Labeled neutrophils were incubated with 10% (vol/vol) SaS or RPMI for 30 min at 37°C on a shaker (400 rpm). After incubation, the cells were washed once and suspended in Hanks balanced salt solution (HBSS)–1% HSA at 2.5×10^6 cells/ml. The upper compartment of the Transwell system was filled with 100 μl of cells and placed into a 24-well microtiter plate containing 600 μl of control buffer or chemoattractant (fMLP, 10^{-7} M; C5a, 1 ng/ml; IL-8, 10^{-8} M). After incubation in a 37°C, 5% CO_2 incubator for 30 min, the Transwell inlays were removed and the fluorescence of the wells was read in the Cytofluor™ multiwell fluorometer (PerSeptive Biosystems, Framingham, Mass.) with an excitation, (485 nm) and emission (530 nm) filter. Control wells of only BCECF-labeled cells were included for the maximal fluorescence value (100%). The fluorescence values of the samples were expressed relative to the total value, yielding the percentage of cells that migrated across the membrane. The fluorescence signal is linear with cell number as determined by serial dilution of the BCECF-labeled cells with a detection limit of 2,500 cells (equals 1% of the added amount). During the incubation, there was no loss of fluorescent label from the cells.

RESULTS

Receptor profile of neutrophils. To study the dynamics and conditions for neutrophil receptor modulation by SaS, we used neutrophils isolated by standard Histopaque-Ficoll procedure. A panel of specific MAbs was used to determine the neutrophil receptor profile after incubation with 10% SaS. This panel contained antibodies against receptors which are involved in immunomodulating processes, like the complement receptors (CR1, CR3, and C5aR), the Fcγ receptors II and III (CD32 and CD16), receptors for adhesion molecules like L-selectin (CD62L) and ICAM (CD11a), phagocyte chemoattractant receptors for fMLP, PAF, C5a (CD88), and IL-8 (CD128), and some other receptors which serve as activation markers for inflammation (CD63, CD66b, HLA class1). For simplicity, we will refer to binding of labeled ligand or labeled antibody to its receptor as "receptor expression" hereafter. The expression of most of the receptors tested did not change after SaS treatment except for the expression of the chemokine receptors for fMLP and C5a (CD88) (Fig. 1). The expression of receptors for the chemotactic agents IL-8 and PAF showed no change after incubation with SaS. Previously, we showed that SaS upregulates CR3 (CD11b) on neutrophils in heparinized whole blood. In isolated cells, we did not observe an upregulation of CR3. When whole blood was incubated with 10% (vol/vol) SaS and subsequently analyzed for neutrophil chemokine receptor profile, only the C5a receptor (CD88) showed a 60% reduction in expression, while the fMLP and IL-8 (CD128w) receptors were not changed (not shown).

The reduced expression of the receptors for C5a and fMLP on isolated neutrophils was studied in more detail. Neutrophils were incubated with various concentrations of SaS and analyzed for receptor expression. The expression of both the C5a and fMLP receptors was decreased in a concentration-dependent matter (Fig. 2). The fMLP receptor expressed was more sensitive and required only 3% SaS for a 50% reduction. Addition of neither phenylmethylsulfonyl fluoride nor EDTA to SaS changed the receptor expression profile for fMLP, C5a, or IL-8 (not shown). The reduction of fMLP receptor expression was not affected by heating the supernate for 30 min at 56 or 100°C. Treatment of the supernate with trypsin or proteinase K (1 mg/ml) did abolish the effect of SaS on fMLP receptor expression (not shown). Time course experiments showed almost immediate effects of SaS on the receptor expression. SaS was not toxic for the cells, as indicated by the lack of change of other receptors. In addition, viability was directly determined with propidium iodide and trypan blue dye exclusion by flow cytometry and microscopy, respectively. Viability of the cells incubated with 50% SaS for 60 min was not affected as compared with control cells and was >95%.

Thirteen other blood isolates of *S. aureus* were tested to see if their supernates were able to downregulate the fMLP receptor on neutrophils as well. About one-third of these strains showed a decreased expression of the fMLP receptor on neutrophils after preincubation with these supernates.

Functional consequences of receptor downmodulation. (i) Calcium flux. Chemokine receptors are G-protein coupled and induce a rapid and transient calcium flux in neutrophils when stimulated. Firstly, a direct calcium flux by SaS was observed with a concentration of 10% (vol/vol) or higher, which was rapid and back to baseline within 2 min. The maximal response was somewhat lower compared to an optimal dose of fMLP (10^{-7} M) or C5a (1 ng/ml). Next, cells were incubated with different amounts of SaS for 10 min at room temperature, after which the calcium levels were recuperated to initial basal levels again. These treated cells were subsequently triggered with an optimal concentration chemoattractant and measured over time to determine the calcium fluxes. Buffer-pretreated cells were run in parallel. SaS concentration dependently prevented the fMLP- and C5a-induced calcium fluxes (Fig. 3). These results are in agreement with the previous data concerning receptor expression.

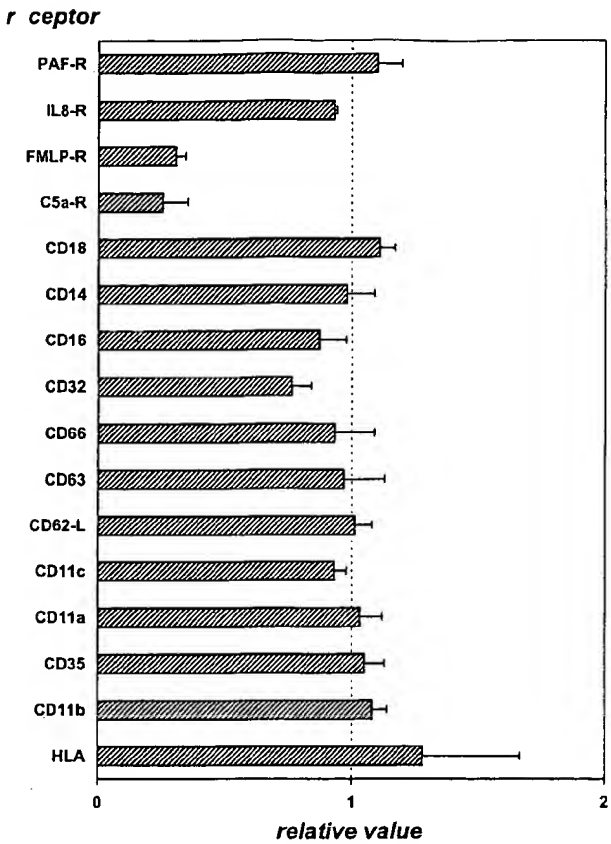

FIG. 1. Receptor expression on neutrophils after 30 min of incubation with 10% (vol/vol) SaS. Data are expressed as relative values compared to those of control cells incubated with buffer. The data represent the means and standard errors of the means of five separate experiments.

(ii) Chemotaxis in vitro. A decrease in receptor expression as measured by fluorescent-antibody binding could be limited to a loss of only the epitope recognized by that specific antibody. That can be the result of masking or conformation change of that specific epitope or loss of the complete antigen. The fMLP receptor expression was actually determined by binding of its (fluorescent) ligand, thereby indicating that its function was affected. Therefore, we not only determined the expression of the receptors but also looked at whether the incubation of the cells with SaS would have consequences for the chemotactic ability of the neutrophils. Mediated C5a chemotaxis was measured by the under-agarose technique and was diminished by SaS in a dose-dependent fashion (not shown). In the Transwell system assay, the chemotactic response of fluorescent-labeled neutrophils incubated with SaS towards fMLP and C5a was abolished, while chemotaxis towards IL-8 was not altered (Fig. 4). These results are in agreement with the data on receptor expression and in addition rule out nonspecific effects of SaS on the neutrophils. In parallel with receptor downmodulation and calcium flux, SaS concentration dependently diminished chemotaxis towards fMLP and C5a (Fig. 5). SaS itself in the lower compartment was slightly chemotactic for neutrophils, but only at a 100% concentration.

DISCUSSION

Directed migration of neutrophils to a focus of infection is an important step in the eradication of invading bacteria by phagocytosis and intracellular killing. A variety of chemoattractants are known to react with specific receptors on phagocytes. Ligation of different chemokine receptors not only initiates chemotaxis but also activates or primes other functions of phagocytes.

Several chemoattractants are known to induce directed migration of neutrophils, including lipid mediators like PAF and leukotriene B4, formylated peptides, activated complement components C3a and C5a, and the chemokines IL-8, NAP, and GRO. Chemoattractants can originate from the bacteria themselves, such as the formylated proteins. Rot et al. purified an fMLP-like tetrapeptide with high chemotactic potency from culture fluids of *S. aureus* (17). Also, bacteria can indirectly generate chemoattractants either via activation of complement resulting in complement fragments with chemotactic activity, such as C3a and C5a, or via activation of cells which in turn produce chemokines. Yao et al. described IL-8 mRNA and protein expression in *S. aureus*-infected endothelial cells (25).

These chemoattractants react with specific receptors that all belong to the family of seven transmembrane "serpentine" receptors that are coupled to G proteins for signaling. The formylated proteins, exemplified by the synthetic tripeptide fMLP, are long recognized as bacterial derived products that induce neutrophil migration by binding to a specific receptor, the fMLP-R. Ligation of fMLP-R not only induces migration but also triggers the generation of oxygen radicals and causes degranulation (24). In turn, it initiates the production of lipid mediators like PAF and LTB4 that are chemoattractants for neutrophils themselves.

We describe here that the supernate of growing *S. aureus* contains a factor(s) that specifically reduce(s) binding of fMLP and αC5a to their receptors on neutrophils, resulting in a strongly attenuated chemotaxis. Staphylococci release several products into the extracellular environment that potently modulate immune cells, including neutrophils. As described for

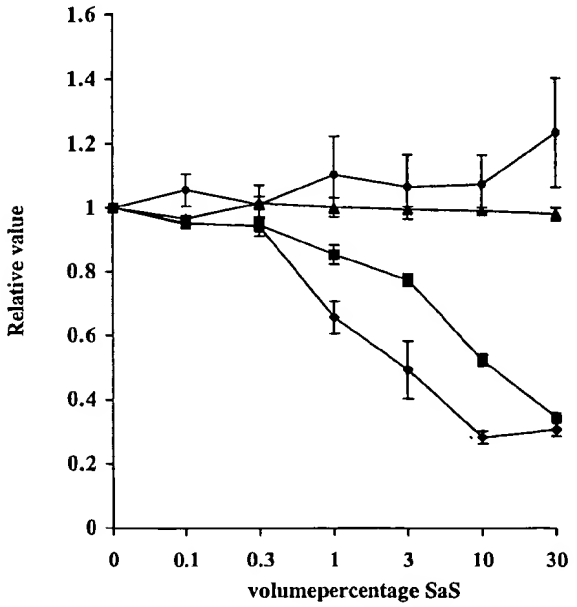

FIG. 2. Chemokine receptor expression (, PAF-R; ▲, IL-8-R; ■,C5a-R; ◆, fMLP-R) on neutrophils after 30 min of incubation with different concentrations (vol/vol) of SaS. Data are expressed as relative values compared to data of controls when neutrophils were not incubated with SaS. The data represent the means and standard errors of the means of three separate experiments.

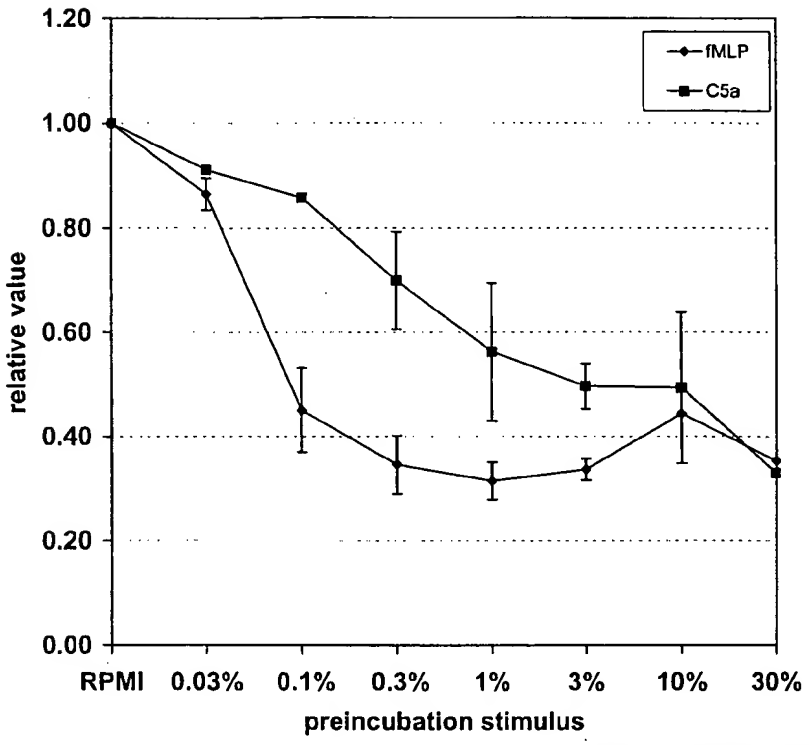

FIG. 3. Calcium flux in neutrophils stimulated with either 10^{-7} M fMLP (◆) or 1 ng of C5a per ml (■) after preincubation for 10 min with different concentrations of SaS. Data are expressed as relative values compared to data of control cells incubated with buffer only.

several bacteria, low-molecular-weight formylated proteins (or peptides) that have chemotactic potency are found in the supernate of *S. aureus*. Because fMLP is an efficient agonist for neutrophil activation, screening of bacterial supernates for neutrophil modulation was performed with SaS devoid of low-molecular-weight components by prior dialysis. Incubation of isolated neutrophils with the dialyzed SaS resulted in a strong attenuation of the expression of receptors for fMLP and C5a. The phenotype of cells incubated with SaS did not resemble that of activated cells, meaning that CD11b expression was not upregulated and no concurrent shedding of L-selectin was observed. In general, activation of neutrophils by many agonists leads to a change in these two receptors, as shown, for instance, for tumor necrosis factor or endotoxin. Moreover, the expression of neutrophil activation markers like CD66 and CD63 was also not changed. Direct toxic effects were not likely, since viability of cells treated for 1 h with 50% (vol/vol) SaS was not affected. Thus, SaS induces specific downmodulation of fMLP and C5a receptor expression, both receptors involved in neutrophil migration. SaS did not change the expression of two other chemoattractant receptors on neutrophils, the PAF-R and IL-8-R, as measured with specific MAbs. The fMLP receptor was more sensitive to SaS treatment than the C5a receptor, but for both molecules, expression was dose dependent.

In whole blood, the effect of SaS on fMLP and C5a receptor expression was less pronounced, probably due to neutralizing components in plasma. In vivo migration takes place after leaving the bloodstream, and staphylococcal infections are mainly located in tissues. Here, concentrations of plasma components are far lower.

Because binding of fluorescent ligand, BODIPY-fMLP, monitored expression of the fMLP receptor, we anticipated that the functional response of the treated neutrophils would also be diminished. SaS efficiently attenuated chemotaxis of neutrophils in a modified Boyden system through a 3-μm-pore-size polycarbonate membrane. The dose response for diminished chemotaxis correlated well with receptor expression as measured by flow cytometry. For the screening of C5a receptor expression, an MAb that binds to an epitope on the N terminus of the C5aR that blocks C5a binding and C5a-mediated acti-

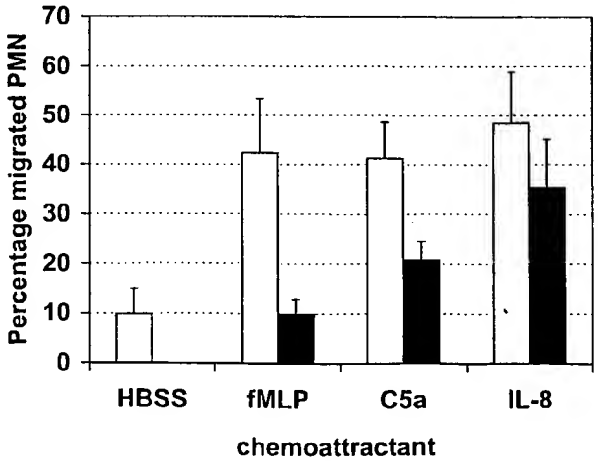

FIG. 4. Chemotaxis of neutrophils towards 10^{-7} M fMLP, 1 ng of C5a/ml, and 10^{-8} M IL-8 in a Transwell system after preincubation for 30 min with 10% (vol/vol) SaS (black bars) or only with buffer (white bars). Data are expressed as the percentage of migrated neutrophils off cells added to the upper compartment. The data represent the means and standard errors of the mean of three separate experiments.

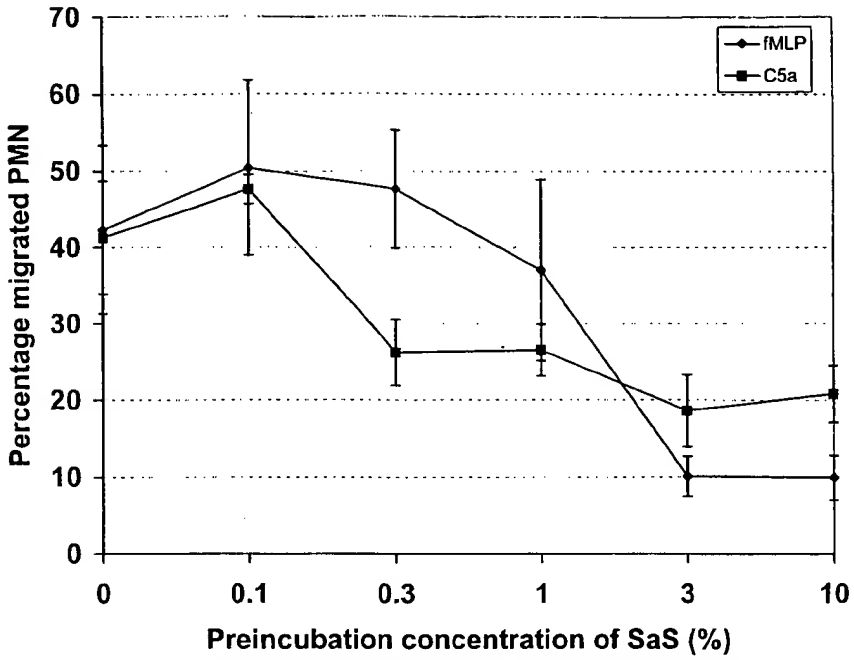

FIG. 5. Concentration-dependent inhibition of chemotaxis of neutrophils towards 10^{-7} M fMLP (◆) and 1 ng of C5a/ml (■) in a Transwell system after preincubation for 30 min with different concentrations of SaS. Data are expressed as the percentage of migrated neutrophils off cells added to the upper compartment. The data represent the means and standard errors of the means of three separate experiments.

vation was used. Therefore, a diminished binding of this antibody to the cells is indicative of a diminished reactivity of the receptor for C5a. Treatment of cells with SaS also concomitantly attenuated the chemotaxis towards recombinant C5a in a dose-dependent way. Directed migration towards IL-8 was not affected in cells treated with SaS, indicating that the effects of SaS were highly specific. In addition, this shows that cells do respond normally to other agonists and are not paralyzed in their locomotory machinery by possible toxic compounds present in SaS.

The family of chemokine receptors that includes the fMLP-R, C5aR, and IL-8-R (CXCR1 and CXCR2) are G protein coupled and are characterized by a rapid and transient rise in intracellular calcium concentration ($[Ca]_i$) upon triggering. Incubation of neutrophils with SaS up to a concentration of 10% initiated a small but significant rise in $[Ca]_i$. Pretreatment of cells with SaS and subsequent triggering with an optimal concentration of fMLP or C5a resulted in a dose-dependent abrogation of the rise in $[Ca]_i$. The calcium response of cells to IL-8 was not affected by SaS. Chemokine receptors show the phenomenon of receptor desensitization that can be the result of both homologous and heterologous ligands. It is known that the receptors for fMLP and C5a show mutual cross-desensitization as measured by ligand binding, receptor recycling, and $[Ca]_i$. Tomhave et al. described efficient receptor cross-desensitization that was restricted between several protein chemokine receptors as well as restricted between lipid chemokine receptors. No cross-talk between protein and lipid receptors was shown (21). In that study, it was also shown that triggering the fMLP or C5a receptor also effectively blunted the response to IL-8 and vice versa. Since SaS did not affect the neutrophil response to IL-8, in neither receptor expression, chemotaxis, nor $[Ca]_i$, it is unlikely that SaS acts directly as an agonist of either the fMLP or C5a receptor. Therefore, we believe that in the supernate of *S. aureus*, one or more products may have an antagonistic effect on both the fMLP and C5a receptors and do not affect the IL-8 receptor on human neutrophils. This results in an abrogated chemotactic response of the cells towards a focus of *S. aureus*.

Modulation of the chemotactic response of neutrophils has been described for several microbial products. Some important studies in this area include the study of Rozdzinski et al., who found that pertussis toxin subunits S2 and S3 of *Bordetella pertussis* share amino acid sequence similarity with the lectin domains of the eukaryotic selectin family and competitively inhibited adherence of neutrophils to endothelial cells in vitro. Intravenous administration of these peptides to animals with meningitis disrupted recruitment of leukocytes into the cerebrospinal fluid (18). The pertussis toxin itself is known to inhibit G-protein-linked receptors like the serpentine receptors by ADP-ribosylation of the G protein (8, 15). *Porphyromonas gingivalis* produces a proteinase which is able to cleave the C5a receptor on neutrophils, thereby inhibiting the chemotactic function towards them (7). Other microorganisms prevent neutrophil influx by interfering with the chemoattractants directly, like most strains of group B streptococci, which elaborate a cell surface-associated C5a-ase that rapidly inactivates the human complement-derived chemoattractants C5a and C5adesarg (2, 6). Glucuronoxylomannan (GXM) of *Cryptococcus neoformans* has been postulated by several authors to prevent neutrophil migration towards chemokines like fMLP, C5a, and IL-8 (3, 4). Lipovsky et al. suggested that cross-desensitization of the receptor might be the reason for the inhibition of neutrophil migration towards IL-8 in the cerebrospinal fluid of infected brain (M. M. Lipovsky, L. J. van Elden, A. M. Walenkamp, J. Dankert, and A. I. Hoepelman, Letter, J. Infect. Dis. **178**:1231–1232, 1998).

The nature of the factor(s) in the staphylococcal supernate responsible for the interference with the fMLP and C5a receptor is subject to further study. Since addition of trypsin as well as proteinase K abolished the effect of SaS on fMLP receptor expression, the responsible factor in SaS is probably a protein

which is heat stable. One could speculate that it might be a protease cleaving off (a part of) the receptor, but we blocked serine protease and metalloprotease activity by adding, respectively, phenylmethyl sulfonyl fluoride and EDTA to SaS in the receptor expression assay without changing the receptor downregulation. Of course, these experiments do not exclude a protease, which has to be checked more thoroughly. Another possibility would be a blocking ligand for these two receptors. Nonspecific general toxic effects of SaS on neutrophils are unlikely since the cells still excluded trypan blue and propidium iodide and not all receptors were prone to change by SaS. To our knowledge, there are no data of a bacterial product which is able to specifically downregulate these two receptors involved in chemotaxis.

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Infection and Immunity, (October, 2000) Vol. 68, No. 10, pp. 5908-5913.

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Effects of granulocyte colony-stimulating factor (G-CSF) treatment on granulocyte function and receptor expression in patients with ventilator-dependent pneumonia

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SUMMARY

Considerable experimental evidence in animals suggests that treatment with G-CSF may have a beneficial effect in the management of severe infections in non-neutropenic hosts. This beneficial effect is attributed to an enhancement of granulopoiesis and neutrophil function, the latter possibly involving up-regulation of receptors on neutrophils that are involved in antibody-mediated cytotoxicity and killing of microorganisms. We compared neutrophil function and phenotype in blood and bronchoalveolar lavage fluid (BALF) of 10 patients with severe ventilator-dependent pneumonia, at baseline and following initiation of G-CSF treatment as adjunct to standard therapy. G-CSF treatment was associated with three-fold increased blood neutrophil counts at day 3 of treatment compared with baseline counts. Mean serum G-CSF concentration increased from 313 to 2007 pg/ml. After correction for lavage dilution effects, BALF G-CSF levels did not differ significantly from baseline, nor did neutrophil receptor expression (FcγRI, FcγRII, FcγRIII, CR3, and L-selectin) or indicators of neutrophil function such as respiratory burst activity, phagocytosis and killing of *Candida albicans* in BALF or blood. The mortality in this group of patients was 30% and compared favourably to the APACHE II-derived predicted mortality of 60%. We conclude that the possible therapeutic benefit of G-CSF administration in the early phase of severe bacterial pneumonia is not readily explained by its effect on baseline indicators of neutrophil function or receptor expression.

Keywords ventilator-dependent pneumonia G-CSF granulocyte function

INTRODUCTION

Adjunctive treatment strategies are clearly needed to improve outcome in serious and complicated infections. To date, agents that have been evaluated clinically such as MoAbs against endotoxin, tumour necrosis factor (TNF), soluble TNF receptors, and IL-1 receptor antagonists, have all failed to confer a convincing beneficial effect [1]. G-CSF is a haematopoietic growth factor that promotes the growth of polymorphonuclear leucocytes. It is produced by vascular endothelial cells, fibroblasts, monocytes, and macrophages. *In vivo*, G-CSF production is stimulated by a variety of stimuli such as (endo-)toxins, bacteria, fungi and certain viruses. The prophylactic use of recombinant human (rh) CSF (including rhG-CSF) is currently recommended in the setting of cancer chemotherapy, among others to reduce the likelihood of and reduce infectious complications associated with febrile neutropenia in high risk patients. Their therapeutic use, as adjuncts to antibiotics, is to be reserved for neutropenic patients with prognostic factors that are predictive of clinical deterioration, such as pneumonia, hypotension, fungal infection, or sepsis [2]. In healthy volunteers, the function of rhG-CSF-induced neutrophils seems to be at least equal to that of normally produced neutrophils [3]. Apart from promoting granulopoiesis, rG-CSF has also been shown to increase neutrophil survival time [4], superoxide anion production, and chemotaxis [5,6], phagocytosis [5], and lipopolysaccharide (LPS) clearance. Up-regulation of the FcγRI (CD64) and RII (CD32), CR1 (CD35), CD14, TNF, and IL-1 receptors and of several receptors involved in neutrophil adherence like CR3 (CD11b/18) and L-selectin [7–9] occurs together with, and is perhaps in part responsible for, these functional changes. *In vivo*, rhG-CSF has been reported to have a beneficial effect in a variety of animal models of infection [10], including pneumonia models [11,12]. Together, these data are

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considered to justify clinical evaluation of rhG-CSF as an adjunctive treatment for serious infections in the non-neutropenic host [12,13].

rhG-CSF was shown to be safe and well tolerated by patients with severe community-acquired pneumonia (CAP) [14]. In a recently concluded phase III study, involving 765 non-neutropenic patients with CAP, treatment with rhG-CSF was associated with a significant reduction in the incidence of ARDS, of diffuse intravascular coagulation, of empyema formation, and with a higher rate of complete resolution of chest x-ray abnormalities by day 28, compared with placebo. However, mortality was not significantly different between treatment arms [15]. Here, we report the results of an open study investigating the effect of rhG-CSF treatment on the expression of receptors and on the function of neutrophils isolated from blood and in bronchoalveolar lavage fluid (BALF) of 10 patients with severe ventilator-dependent pneumonia. The aim of this study was to document rhG-CSF-associated changes in baseline levels of neutrophil function and receptor expression that might explain some of the presumed beneficial effects of rhG-CSF in the study patients with severe CAP [15].

SUBJECTS AND METHODS

Patients

The study protocol was approved by the institutional review board of Utrecht University Hospital. Inclusion required written informed consent from patients or their relatives. Patients requiring mechanical ventilation for a presumed diagnosis of pneumonia, according to the criteria adopted by the Infectious Diseases Society of America [16], were eligible for inclusion. The following exclusion criteria applied: < 18 years of age, pregnancy, enrolment in any other investigational protocol, any concomitant end-stage disease, neutropenia (< 500 neutrophils/ml), concurrent immunomodulatory treatment other than non-steroidal anti-inflammatory drugs or corticosteroids, documented hypersensitivity to rhG-CSF, or mechanical ventilation for more than 24 h following a presumed diagnosis of pneumonia. Healthy volunteers were donors of blood from which neutrophils were isolated for use as controls in the *ex vivo* experiments described below.

rhG-CSF

Commercially available recombinant-methionyl G-CSF (filgrastim; Neupogen) was used. Patients received once daily subcutaneous injections of 300 μg (if < 70 kg body wt) or 480 μg (if $\geq$ 70 kg body wt) until off-ventilator, with a maximum of 14 days. Administration of G-CSF was to be suspended once the leucocyte count exceeded 60 000 cells/ml. Administration of reduced (50%) doses was to be resumed when the leucocyte count had subsequently fallen to $\leq$ 30 000 cells/ml.

Bronchoalveolar lavage

Using a flexible fibreoptic bronchoscope wedged in the affected lung segment, bronchoalveolar lavage (BAL) was performed by instillment and subsequent recovery of an aliquot of 20 ml normal saline, followed by three consecutive aliquots of 50 ml normal saline. The mean recovery was 48% (range 28–86%). The first aliquot was discarded, whereafter all subsequently recovered BALF was pooled in a silicone-lined bottle. These samples were immediately centrifuged (300 *g*, 10 min) and supernatants were stored at −20°C until further use. Pellets were gently resuspended in a small volume of RPMI, containing 0·05% human serum albumin (HSA) (RPMI/HSA) and further processed for cell counting, differentiation, and neutrophil function tests as described below.

Cells

Blood neutrophils were isolated from heparinized blood by Ficoll density centrifugation as previously described by us [17].

Neutrophil chemiluminescence

The oxidative burst capacity and the priming state of neutrophils, as a measure of cell activation, were measured in whole blood and resuspended BALF cells, using a chemiluminescence assay. Tubes precoated with different priming agonists (C5a, platelet-activating factor (PAF), fMLP; ExOxEmis) were mixed with C3b-opsonized Zymozan and placed into the transport chain of a Berthold luminometer (Autolumat LB 953). EDTA blood or BALF cells were diluted 100-fold with PBS containing 5% glucose. Prewarmed luminol solution (Hanks' balanced salt solution (HBSS) with 0·05% HSA and 150 μM luminol), lucigenin solution (ExOxEmis), and diluted blood were automatically injected into the tubes, and each tube was repeatedly measured for 20 min at 37°C. The ct/min were recorded and, for each sample, calculated as total counts over a 20-min period. The values obtained were corrected for the absolute neutrophil counts (ANC), as determined by electronic counting (CoulterCounter) and a (Diff Quick-stained) blood smear or cytospin preparation.

Neutrophil chemotaxis

Directed migration of isolated neutrophils towards Zymosan-activated serum was determined using the under agarose technique [18].

Neutrophil receptor expression

The expression of several neutrophil receptors was studied in whole EDTA-treated blood and in resuspended BALF cells by direct immunofluorescence with specific MoAbs: MoAb 22–FITC (anti-CD64, FcγRI), IV.3–FITC (anti-CD32, FcγRII), and 3G8–FITC (anti-CD16, FcγRIII) were obtained from Medarex; MoAb 44a–FITC (anti-CR3) was prepared in our laboratory from an ATCC hybridoma; MoAb Leu-8–FITC (anti-L-selectin) was obtained from Becton Dickinson (Mountain View, CA). Fifty microlitres of blood or resuspended BALF cells were mixed with saturating amounts of MoAb and kept on ice for 30 min. Samples were then lysed and fixed with lysis buffer (Becton Dickinson), centrifuged (10 min, 300 *g*), washed once with ice cold RPMI/HSA, and again centrifuged. The cell pellet was resuspended in 0·5% paraformaldehyde and the sample was analysed on a FACScan flow cytometer (Becton Dickinson). Samples of 10 000 cells were analysed for neutrophil-associated fluorescence by appropriate gating of forward- and side-scatter parameters to exclude cell debris, large aggregates, and other cell types from the analysis.

Phagocytosis

The phagocytic capacity of isolated blood neutrophils and of neutrophils in BALF was measured with FITC-labelled *Staphylococcus aureus*, Cowan EMS, and flow cytometry, as previously described by us [17]. Bacteria and cells were mixed at a ratio of 10:1 in the presence of different concentrations of pooled human serum. After 15 min the reaction was stopped by fixation, and phagocytosis was expressed as the percentage of neutrophils with

Table 1. Clinical characteristics of 10 patients with severe ventilator-dependent pneumonia, treated with recombinant human (rh)G-CSF

Sex	Age	APACHE II	Premorbid conditions	Sputum culture*	Days on rhG-CSF	Disease	Outcome course (day 28)
F	71	40	Rheumatoid arthritis	*S. aureus*	14	Septic shock, MODS, therapy withdrawn	Died
M	72	29	Alcohol abuse/cranial trauma	*S. aureus*	5	Uneventful recovery	Alive
F	42	27	Healthy	*S. pneumoniae*	7	Fulminant *S. pneumoniae* sepsis, septic shock, MODS†	Died
M	74	19	Ruptured aortic aneurysm, COPD	*E. coli*‡		Uneventful recovery	Alive
M	40	15	Alcohol abuse/polyneuropathy	*S. aureus*	7	Uneventful recovery	Alive
M	39	31	Epileptic status; resuscitated	*C. koseri*	7	Post-anoxic encephalopathy, therapy withdrawn	Died
F	43	28	Cranial trauma	*P. aeruginosa*‡	7	Uneventful recovery	Alive
M	42	28	Alcohol abuse/acute pancreatitis	*P. aeruginosa*‡	14	Sepsis, MODS, emergency surgery for spontaneous splenic artery rupture with haemorrhagic shock	Alive
M	60	30	ChemoTx for ENT malignancy	*E. coli*‡	14	Uneventful recovery	Alive
M	54	28	Malignant neuroleptic syndrome	*P. aeruginosa*‡	13	*P. aeruginosa* bacteraemia, septic shock	Alive

* Culture was done if sputum contained < 10 epithelial cells and ≥ 25 PMN per low-power field (×10).
† Multiple organ dysfunction syndrome.
‡ Hospital-acquired pneumonia.

associated FITC-labelled bacteria. We did not discriminate between internalized and attached bacteria.

Killing of Candida albicans

Using flow cytometry, the killing capacity of isolated neutrophils was determined by the ability of *Candida* to retain a fluorescent probe. This technique was previously used by our laboratory for *Cryptococcus neoformans* [19]. Briefly, a clinical isolate of *C. albicans* was grown overnight in selective medium containing 6 μM 2,7-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein, acetomethyl ester (BCECF-AM), washed, and stored at −70°C in PBS containing 20% glycerol. An aliquot was thawed, washed, and spectrophotometrically adjusted to 1×10^7 cells/ml. *Candida albicans* was mixed with isolated neutrophils in a ratio of 3:1 and incubated for 30 min in the presence or absence of 3% normal serum at 37°C under shaking. An aliquot was taken from each tube and mixed with an equal volume of deoxycholate (25 mM) for 4 min at room temperature to solubilize the neutrophils. Then the samples were fixed with an equal volume of 1% paraformaldehyde and analysed in a flow cytometer for Candida-associated fluorescence. After exclusion of cell debris and cell aggregates, the fluorescence histogram of the *Candida* population was divided into viable, fluorescent cells and killed non-fluorescent cells (background fluorescence). The percentage of killing was corrected for the spontaneous loss of probe when *C. albicans* was incubated in medium alone.

Other laboratory assessments

Serum and BALF G-CSF levels were determined by a commercial ELISA system (Quantikine human G-CSF Immunoassay test kit; R&D Systems, Minneapolis, MN; lower limit of detection 39 pg/ml). Blood biochemistry and haematology parameters were measured by routine laboratory techniques. The BALF urea content was determined colourimetrically in urease-treated samples. To correct for lavage dilution effects, the results of BALF G-CSF assays were multiplied by the serum/BALF urea ratio [20].

Statistical analysis

Pretreatment and on-treatment continuous variables were compared by two-sample *t*-test. $P \leq 0.05$ was considered to denote a significant difference.

RESULTS

Patients

Fourteen of 16 consecutive patients requiring mechanical ventilation for severe pneumonia were eligible for inclusion. Ten patients consented to enter the study. Their mean (range) age was 54 years (39–74 years), their mean pretreatment APACHE II score was 27 (15–40), the mean baseline PaO_2/FiO_2 ratio 200 (104–356), and the mean number of days until off-ventilator was 10 (5–21). Nine patients had premorbid underlying conditions considered to increase their risk of acquiring serious infections. Three patients developed septic shock with multiple organ dysfunction. The all-cause mortality at day 28 after initiation of rhG-CSF treatment was 30%. Treatment was withdrawn in two patients. The mean APACHE II-derived predicted 28-day mortality [21] was calculated to be 60%. Bacterial pneumonia was culture-documented in all patients and considered hospital-acquired in five (50%) of them. Sputum cultures of six patients yielded single isolates of *S. aureus* ($n = 3$) and *Pseudomonas aeruginosa* ($n = 3$). Patients received rhG-CSF for a mean of 10 days (range 5–14 days). There were no noticeable adverse effects of rhG-CSF treatment in any patient. In only one patient was rhG-CSF administration suspended because of a neutrophil count exceeding 60 000 cells/ml. Details of the clinical and demographic characteristics are shown in Table 1.

Blood and BALF neutrophil counts and G-CSF levels

Mean blood neutrophil counts had tripled by day 3 of G-CSF treatment (27.9×10^6 cells/ml, range $7.4–57.2 \times 10^6$ cells/ml) and peaked, virtually without exception, by day 7 (mean 45×10^6 cells/ml, range $28.6–96.5 \times 10^6$/ml) (Fig. 1). The mean percentage (range) of band forms was 12·6 (1–30) at baseline and 11·9

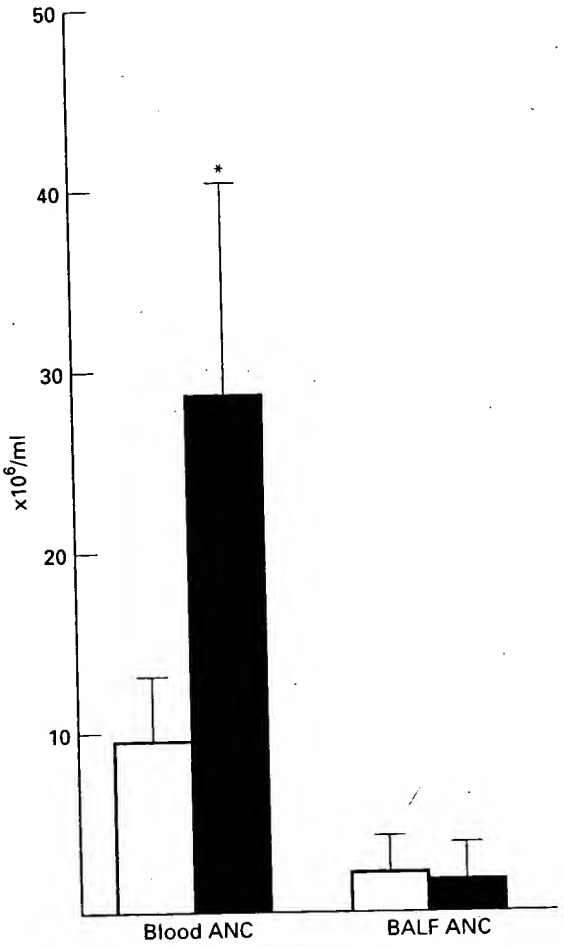

Fig. 1. Mean blood and bronchoalveolar lavage fluid (BALF) neutrophil counts in 10 patients with ventilator-dependent bacterial pneumonia before (day 0; □) and following the start of recombinant human (rh)G-CSF treatment (day 3; ■). Error bars indicate s.e.m. *$P<0.001$. ANC, Absolute neutrophil counts.

Table 2. G-CSF levels in serum and bronchoalveolar lavage fluid (BALF) of patients with ventilator-dependent pneumonia before and on the third day after the start of treatment with recombinant human G-CSF

	Serum G-CSF (pg/ml)		BAL G-CSF (pg/ml)	
Patient no.	Day 0	Day 3	Day 0	Day 3
1	NA	4313	8008	4323
2	441	2673	936	850
3	NA	265	10016	6295
4	NA	NA	NA	NA
5	NA	NA	1155	NA
6	129	426	NA	561
7	215	39	6369	15719
8	182	NA	5330	339
9	NA	NA	1014	2939
10	599	4325	NA	NA
Mean ± s.e.m.	113 ± 165	2007 ± 1764	4690 ± 3132	4432 ± 3757

NA, No data available.

(1–37) on day 3. The mean BALF neutrophil count on day 3 (1.83×10^6 cells/ml) did not significantly differ from that at baseline (2.2×10^6 cells/ml) (Fig. 1). Mean (range ± s.e.m.) serum G-CSF concentrations on day 0 ($n=5$) and day 3 ($n=6$) were 313 pg/ml (182–599 pg/ml; ± 165 pg/ml) and 2007 pg/ml (39–4325 pg/ml; ± 1764 pg/ml), respectively ($P<0.05$). In BALF, these concentrations were 4690 pg/ml (937–10 016 pg/ml; ± 3133 pg/ml) and 4432 pg/ml (339–15 719 pg/ml; ± 3757 pg/ml), respectively, after correction for dilution effects (Table 2).

Phagocytosis, oxidative burst, and chemotaxis

Without exception, phagocytosis by blood neutrophils of patients was increased, but not significantly, compared with that of neutrophils from untreated healthy controls, both at baseline and at day 3. Compared with baseline, no significant change in the level of phagocytosis by neutrophils in blood or BALF was observed on day 3 (Fig. 2). However, at baseline and on day 3, in the presence of low opsonin concentrations, blood and BALF neutrophils from patients showed higher levels of phagocytosis than did neutrophils from controls. The oxidative burst activity of neutrophils (both in blood and BALF) did not differ significantly between day 0 and day 3 of G-CSF treatment (not shown). The mean chemotactic activity of neutrophils from the blood of patients decreased significantly ($P<0.05$) from baseline to day 3, but was not significantly different from that of neutrophils from healthy controls, either at baseline or on day 3 of rhG-CSF treatment. Polymorphonuclear neutrophils (PMN) in BALF did not show chemotactic activity.

Killing of Candida albicans

Neutrophils isolated from the blood or in BALF of patients had a (non-significant) decreased ability to kill *C. albicans* compared with that of neutrophils from controls, both before and during G-CSF treatment (Fig. 3).

Neutrophil receptor expression

Isolated neutrophils from patients and healthy controls were evaluated for receptor expression before (day 0) and during (day 3) rhG-CSF treatment. The following receptors were studied: FcγRI, FcγRII, FcγRIII, CR3, and L-selectin. No significant differences were observed on day 3 compared with baseline levels. Baseline expression of FcγRI, FcγRII, and CR3 receptors tended to be higher in neutrophils isolated from BALF than in neutrophils isolated from the blood of patients and healthy donors (controls), probably indicating that the cells were primed. Expression of the CR3 receptor on BALF neutrophils was significantly higher than that on blood neutrophils from patients and healthy controls on day 3 of treatment. On day 3, FcγRI receptor expression on neutrophils in BALF and blood from patients was significantly increased compared with that of controls ($P<0.05$). The expression of FcγRIII and L-selectin receptors tended to be lower in neutrophils from patients (both in BALF and blood) than in neutrophils from controls. These differences were not significant (Fig. 4).

DISCUSSION

In the present study we have shown that rhG-CSF treatment of patients requiring mechanical ventilation for severe pneumonia was well tolerated and associated with a three-fold increased mean blood neutrophil count on day 3 of treatment, compared with

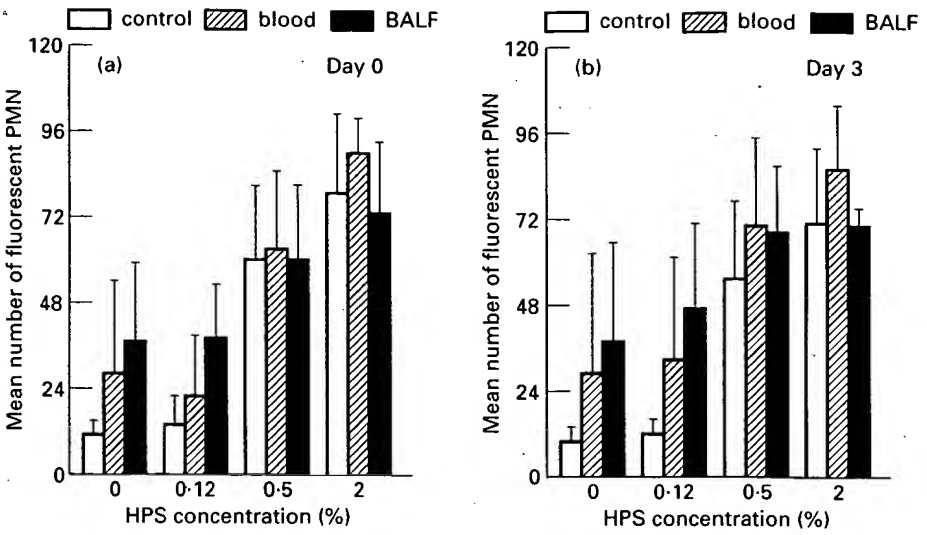

Fig. 2. Phagocytosis by neutrophils isolated from blood or in bronchoalveolar lavage fluid (BALF) obtained from 10 patients with ventilator-dependent bacterial pneumonia before (day 0) (a) and following the start of G-CSF treatment (day 3) (b). Neutrophils were incubated with FITC-labelled *Staphylococcus aureus*, in the absence or presence of graded concentrations of human pooled serum (HPS). Phagocytosis was quantified by the number of fluorescent neutrophils. Neutrophils isolated from blood of healthy untreated donors served as control. Error bars indicate s.e.m. No significant differences were observed.

baseline counts. The number of neutrophils in BALF was not affected by rhG-CSF treatment. Neither the expression of neutrophil FcγRI, -RII, and -RIII, CR3, or L-selectin receptors on neutrophils, nor the studied indicators of neutrophil function (phagocytosis, oxidative burst, chemotaxis, killing of *C. albicans*) on day 3 of rhG-CSF treatment were significantly different from corresponding baseline values. These findings contrast with the significantly increased levels of FcγRI receptor expression following rhG-CSF treatment reported by others [22,23]. However, they investigated neutrophils in healthy volunteers or in non-infected patients, which may explain the much larger difference between pretreatment and treatment levels of FcγRI expression. This hypothesis is supported by the fact that baseline (endogenous) G-CSF concentrations (313 ± 165 pg/ml) were significantly elevated in our patients compared with values reported for healthy volunteers and non-infected patients [24]. The higher G-CSF concentrations in BALF compared with blood, as found at baseline, may be related to the local production of G-CSF by alveolar macrophages [25]. Elevated endogenous G-CSF concentrations have been reported to accompany the acute phase of bacterial infection [26]. We previously reported similar findings in a study with murine anti-TNF MoAb in patients with sepsis syndrome and septic shock [27]. In this study, baseline mean (± s.d.) endogenous G-CSF concentrations ranged from 1507 ± 934 pg/ml in patients with negative blood cultures to 175 607 ± 171 103 pg/ml in patients with documented Gram-negative bacteraemia. Elevated endogenous G-CSF concentrations may have caused the high(er) baseline levels of neutrophil receptor expression and functional activity of neutrophils isolated from our patients, and may explain why no significant differences were observed between values at baseline and on day 3. We do not know if this lack of difference is related to the timing (day 3 of G-CSF treatment) chosen for comparison with baseline. Spiekerman *et al.* [23] reported an enhancement of receptor expression and neutrophil function within 2–4 days of rhG-CSF treatment in patients undergoing chemotherapy for malignancy. Allen *et al.* [28], studying the

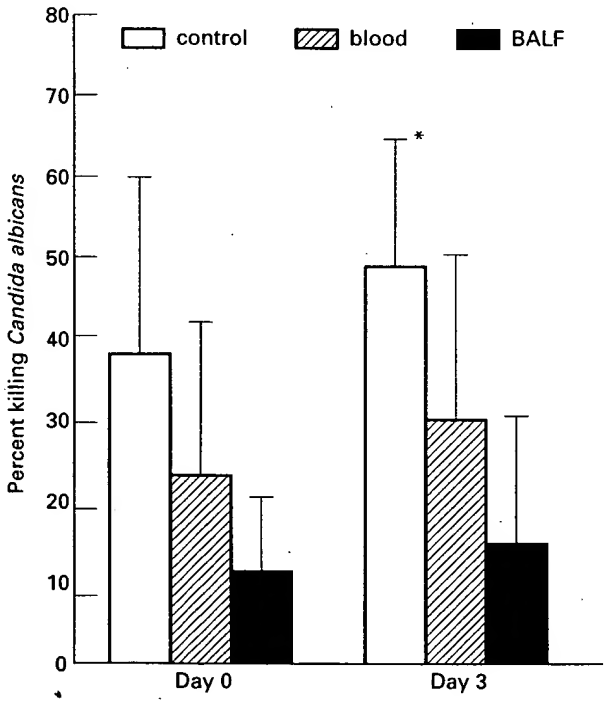

Fig. 3. Killing of *Candida albicans* by neutrophils in blood or bronchoalveolar lavage fluid (BALF) obtained from 10 patients with ventilator-dependent bacterial pneumonia before (day 0) and following the start of G-CSF treatment (day 3). Neutrophils isolated from blood of healthy untreated donors served as control. Error bars indicate s.e.m. No significant differences were observed between day 3 and baseline in corresponding samples. *$P < 0.05$ control *versus* BALF on day 3.

effect of G-CSF administration on neutrophil function and receptor expression in healthy volunteers, reported significantly increased PMN function by day 3 with a maximum effect by day 5 of G-CSF treatment. Our findings suggest that the mechanism of any added

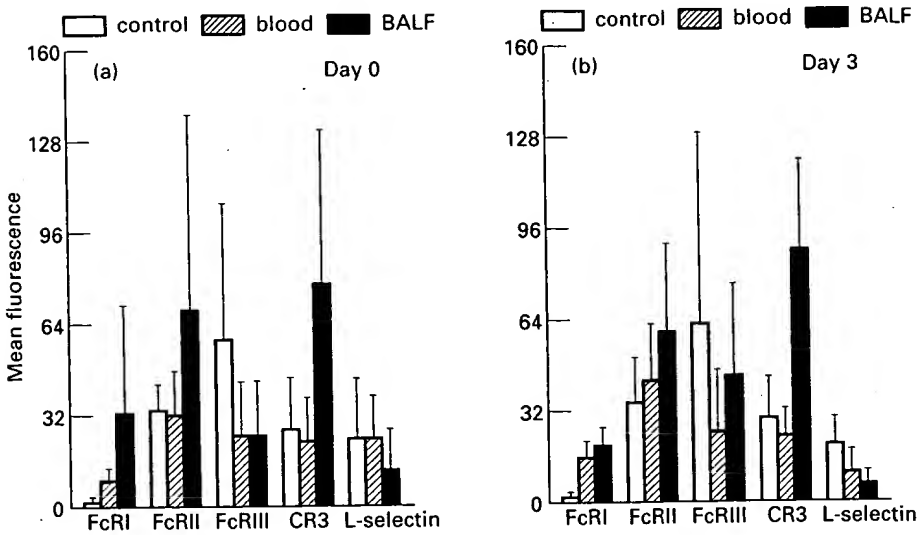

Fig. 4. Mean expression of FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), CR3 (CD11b) and L-selectin (CD62L) receptors on neutrophils isolated from blood and in bronchoalveolar lavage fluid (BALF) from 10 patients with ventilator-dependent pneumonia, before (day 0, (a)) and during (day 3, (b)) treatment with recombinant human (rh)G-CSF. Neutrophils isolated from blood of healthy untreated donors served as control. Error bars indicate s.e.m. No significant differences were observed between pretreatment and treatment levels of expression of individual receptors. $*P < 0.05$ *versus* control; $**P < 0.01$ *versus* blood neutrophils of controls and patients.

protection conferred by concomitant rhG-CSF treatment of patients with acute infections does not involve receptor up-regulation and enhancement of neutrophil function. This leaves the question open by what mechanism the benefit of adjunctive rhG-CSF treatment, as reported in a range of experimental studies under non-neutropenic conditions (reviewed in [10]), is achieved.

In these studies, rG-CSF was efficacious, whether administered prophylactically or therapeutically. Enhanced neutrophil recruitment to the site of infection [29], attenuation of the systemic TNF inflammatory response [29–31] and, possibly, synergism with antibiotic therapy [13,32] are suggested mechanisms for the protective or therapeutic action of rG-CSF. It was initially feared that administration of rhG-CSF to patients with sepsis might lead to or aggravate ARDS, through rhG-CSF-induced enhancement of neutrophil recruitment to sites of infection. However, studies in pneumonia models have provided evidence to the contrary. In our patients, BALF neutrophil counts did not increase with rhG-CSF treatment, although we do not know to what extent such counts adequately reflect changes in the interstitium. A similar finding was reported in bone marrow transplant patients [33]. In a study of neonatal sepsis, rhG-CSF administration was not associated with pulmonary or other organ toxicity [34]. The recent consensus statement by the American Thoracic Society (ATS) [35] has indicated that hospital-acquired pneumonia (HAP) continues to be the number one cause of death from nosocomial infection. On average, the attributable mortality of HAP is estimated to be 25–35%. The associated mortality may, however, increase dramatically to levels of $\geq 70\%$ when HAP is complicated by signs and symptoms consistent with sepsis.

A better understanding of the pathogenesis of severe HAP (and sepsis, for that matter) and additional therapeutic options are clearly needed to improve the outcome of severe HAP and CAP. The ATS consensus statement supports the clinical evaluation of cytokines such as rhG-CSF and interferon-gamma as possible adjuncts to standard therapy for severe HAP. This may also apply to other cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) [13]. The mortality in the group of patients described here was 30% and compared favourably with the APACHE II-derived mean predicted mortality of 60%. We conclude that the possible therapeutic benefit of rhG-CSF administration in the early phase of severe ventilator-dependent pneumonia is not readily explained by its effect on baseline indicators of neutrophil function or receptor expression. Clearly, a blinded placebo controlled study is needed to substantiate this seemingly beneficial effect.

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